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| 13. ABSTRACT (Maximum 200 Words) The focus of this project was to design a system for screening chemical compounds that would inhibit the dimerization of the EGFR family members ErbB2 and ErbB3 using beta-galactosidase complementation. We attempted to apply the beta-galactosidase complementation system to both ErbB2,3 homo and heterodimerization, as well as the interaction of these receptors with downstream signaling molecules such as Grb2. The initial attempts to design an assay amenable to high-throughput screening for antagonists of ErbB2 activity failed due to fundamental problems associated with the interaction system employed. In order to circumvent these limitations we rationally redesigned the system to be more stable and tolerate wider ranges of expression levels. The new system based on a 46aa mutated α -peptide was able to successfully discern inducible interactions for transmembrane receptors, cytosolic, and nuclear proteins. Further the magnitude of induction is sufficient for high throughput screening for drugs that inhibit this heterodimerization event. | | | |
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Introduction

Multiple common carcinomas frequently express high levels of the EGF family of tyrosine kinase growth factor receptors (EGFR/ErbB1, ErbB2, ErbB3). Specifically, ErbB-2 is upregulated in a high percentage of breast cancer tumors. The frequency of ErbB-2 overexpression indicates that this may be an early and often necessary change for successful tumor engraftment. ErbB-2 is a receptor tyrosine kinase which activates multiple intracellular pathways mainly associated with cell proliferation and survival. Recent evidence shows that therapies blocking ErbB-2 signaling are effective anti-cancer agents. The EGFR, and ErbB-3 are also upregulated, although to a lesser degree, in mammary carcinomas. The mechanism by which ErbB-2 receptor mediates its effects is complicated by the ability of the EGF family of receptors to heterodimerize and activate different signaling pathways. The general aims of this research are to develop a high-throughput screen for antagonists of signaling events mediated by the EGF family of receptors, and to identify novel proteins that associate with the receptors as potential drug targets. To accomplish these goals we are applying a technique pioneered in our laboratory based on enzyme complementation of β galactosidase. This approach involves the construction of chimeric proteins consisting of two proteins of interest which dimerize, for example ErbB-2 and the EGFR, and two inactive portions of an enzyme. The dimerization of the proteins forces the complementation of the enzyme resulting in an increase in enzyme activity which can be assayed by fluorescence or light emission and can be performed in 96-well format.

The major limitation of the β -galactosidase system is the high inherent affinity of the two complementing fragments. This affinity makes the system extremely sensitive to protein expression levels. Thus stable cell lines must first be created then clones from this parental line expressing various levels of the fusion proteins tested for their ability to respond to the appropriate stimulus through increases in β -galactosidase activity. The need to find responding clones adds months to the development of the assay and precludes quantitative comparisons between cell lines.

In order to design a system that could dynamically monitor protein interactions in live mammalian cells we started with one of the most efficient complementation reactions known, α -complementation of β -galactosidase. First we minimized the α peptide then mutated it to create a low affinity complementation system. We successfully employed this system to monitor interactions in various cell compartments in heterogeneous populations. Finally we succeeded in applying this system to the heterodimerization of the EGFR and ErbB2.

Body

I. Redesigning the β -galactosidase complementation system

Aside from the large inherent affinity of the two previously isolated fragments, proteins fused to the α -donor were highly unstable. Thus two goals of redesigning the system were to make the α -donor more stable, and make the system tolerant to varying levels of protein expression. The approach we took was to first minimize the α donor then mutate it to generate a low affinity complementation system.

A) Defining the minimal α peptide.

α complementation of β -galactosidase involves a deletion mutant, the ω fragment (ω), that is missing aa 11-41 (Langley KE 1975). In *E. coli* and *in vitro* it is possible to complement this mutant in trans by providing the α peptide that encodes aa 3-92 (Langley KE 1975). However in mammalian cells the smallest complementing fragment was reported to contain aa 1-71 (Moosmann and Rusconi 1996). We sought to determine the smallest α peptide that would complement robustly in mammalian cells when fused to an exogenous protein at the N or C terminus. The myoblast cell line C2C12 was engineered to stably express the ω fragment by retroviral transduction. The α portion of β -galactosidase was fused to the C-terminus of GFP and deletions were made starting at aa 137 and continuing through aa 38. The products were transduced into the parental cell line expressing the ω -fragment and the cells were sorted by FACS for similar GFP expression levels. Cells were plated in 96-well dishes and assayed for β -galactosidase activity using a 1,2 dioxetane luminescent substrate (Fig. 1, right panel). From this experiment it is clear that truncations past aa 49 result in a 25-fold decrease in β -galactosidase activity. To determine which residues could be deleted at the N-terminus, aa 1-51 were fused to the N-terminus of GFP and deletions were made starting at aa 5. These constructs were similarly transduced into the parental C2C12 cell line expressing the ω -fragment. Deletions past aa 5 resulted in a 100-fold decrease in β -galactosidase activity (Fig. 1A, left panel), combining these deletions the minimal complementing fragment in mammalian cells should encompass aa 5-49. To ensure this peptide would complement at the same level when fused to either the amino or carboxyl terminus of a protein, the peptide was fused separately to either end of GFP (Fig. 1C). When the peptide is expressed as a C-terminal fusion robust complementation is achieved, however fusion of the same peptide to the N-terminus resulted in 100-fold less activity when aa 5-49 was used while robust complementation was achieved with aa 5-51. Thus to maintain consistency for the ensuing studies aa 5-51 (designated α) is the minimal fragment that complements to high activity in mammalian cells when expressed as a fusion protein in any orientation.

B) Mutation of the α peptide

The crystal structure of β -galactosidase shows that residues 5-28 of the α peptide are involved in dimerization of two monomers while residues 31-41 are buried within the ω fragment. We reasoned that mutation of the buried residues would decrease the ability of the α peptide to dock with the ω fragment while maintaining its ability to mediate tetramer formation.

We engineered several point mutations spanning residues 31-41 into the α fused to the carboxyl terminus of GFP (Fig. 2A). These constructs were transduced into the tEGFR- ω cell line, sorted for similar amounts of GFP expression, and then assayed for β -galactosidase activity using the luminescent assay. Of the seven mutations tested, five were found to decrease complemented enzyme activity from 2-30-fold (Fig. 2B). Of these the H31R mutant was chosen for further study.

II. Using the H31R mutant to monitor inducible protein interactions

Due to the low background enzyme activity when the H31R mutant is coexpressed with the ω -fragment we reasoned that the modified α -complementation system could be applied to protein interactions. To test this hypothesis, we fused the H31R mutant to FKBP12 and FRB to the N-

terminus of the ω fragment. FKBP12 and FRB have an undetectable affinity for one another, however when the drug rapamycin is present the two proteins heterodimerize efficiently. As shown in Figure 3 the addition of rapamycin to C2C12 cells expressing both fusion constructs results in marked increases in enzyme activity.

Although this demonstrates the utility of the system for protein interactions, the FKBP12-FRB pair is unlike a typical protein interaction due to its low background (-rapamycin) and high affinity (+rapamycin) states. To determine whether the system was robust enough to monitor endogenous protein interactions we engineered four protein pairs that inducibly dimerize in a range of cellular compartments including the cytosol, nucleus, and constrained within the plasma membrane. Figure 3 shows that all protein pairs tested achieved significant interaction-dependent activity in the presence of the appropriate stimulus. The previously described system to monitor protein interactions based on β -galactosidase was extremely sensitive to fusion protein concentration such that clones of stable populations had to be screened in order to find cells that respond. The new system tolerated a wide range of expression levels and provided measurable increases in enzyme activity in heterogeneous populations. This ability vastly decreases the time invested in developing each assays and permits the comparison of cell lines expressing different protein pairs.

Homo and heterodimerization of the EGFR family monitored by enzyme complementation

First we applied the modified β -galactosidase complementation system to the homodimerization of the EGFR. The extracellular and transmembrane domains of the EGFR were fused to the ω and H31R mutant. C2C12 cells were cotransduced with both constructs then assayed for their ability to complement in the presence of EGF. Immediately noticeable in Figure 4 is that the presence of ligand increases β -galactosidase activity by 2-3 fold. In heterogeneous populations, the H31R system outperformed the older version by almost an order of magnitude in signal increase. Next we extended our studies to the heterodimerization of the EGFR and ErbB2. Similarly, in the presence of EGF as a dimerizing agent, a 2-3 fold increase in β -galactosidase activity is achieved. These results indicate that the EGFR and ErbB2 efficiently heterodimerize in a manner that is ligand responsive. Significantly, although the EGFR is known to phosphorylate and activate ErbB2, heterodimerization of the extracellular domains using purified proteins, (Ferguson, Darling et al. 2000) or ^{125}I labeling and crosslinking, or previous attempts using the old β -galactosidase system, failed to show an inducible interaction (Johannessen, Haugen et al. 2001). The ability to detect this EGF dependent heterodimerization event attests to the sensitivity and utility of this technique in monitoring protein interactions. As such this newly designed system to monitor protein interactions should have broad utility in quantitatively defining signal transduction events and in high-throughput screening applications.

Key Research Accomplishments

Rational redesign of the β -galactosidase complementation system: We have re-engineered the β -galactosidase system to be more stable as fusion proteins and tolerate a wider range of protein expression levels. These advances permit use of the system with inducible protein interactions that were previously undetectable.

Utilization of the H31R system to monitor inducible interactions: Using the FKBP12-FRB system we proved it is possible to monitor inducible protein interactions.

Physiological interactions measured using the H31R system: To determine the applicability of the system to lower affinity interactions we used the system to monitor the dimerization of the TGF- β receptors, and the interaction of SMAD2 and SMAD4, both of which are TGF- β responsive. The H31R system succeeded in both cases to generate significant increases in enzyme activity, demonstrating its utility in monitoring interactions at the plasma membrane, cytosol, and nucleus.

Homo and heterodimerization of the EGFR and ErbB2 successfully detected using the H31R mutant: In a polyclonal population of cells the homodimerization of the EGFR and heterodimerization of the EGFR and ErbB2 were successfully assayed in the presence or absence of EGF ligand using the H31R system.

Reportable Outcomes (for entire project)

Ph.D. Thesis:

Wehrman, Thomas S. (May 2004) Development of protein complementation assays and small interfering RNA libraries for protein interactions, localization, and gene function. Ph.D. Thesis, Stanford University, Stanford, CA.

Manuscripts:

Wehrman, T.S. and Blau, H.M. (2004) Protein localization and interaction monitored by modified α -complementation of β -galactosidase, manuscript in preparation (see appendix I).

Blakely, B.T., Rossi, F.M.V., Wehrman, T.S., Charlton, C.A. and Blau, H.M. (2002) Protein interactions in live cells monitored by β -galactosidase complementation. In Protein-Protein Interactions: A Molecular Cloning Manual, (E. Golemis, ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 407-414 (copy previously submitted, so not included in appendix).

Wehrman, T.S., Kleaveland, B.M., Her, J-H., Balint, R. and Blau, H.M. (2002) Protein-protein interactions monitored in mammalian cells via complementation of β -lactamase enzyme fragments. Proc. Natl. Acad. Sci. USA 99:3469-3474. (copy previously submitted, so not included in appendix).

Patent: (listed in previous report)

1997: Detection of molecular interactins by reporter subunit complementation, US Patent No. 6,342,345 B1 – International Application No. PCT/US98/06648 – Stanford Docket S96-125 (issued 1/29/02)

Constructs: Retroviral vectors containing truncated ErbB (tErbB) family members and a resistance gene (listed in a previous report)

pWZL-tErbB1- $\Delta\omega$ IRES-Hygromycin
pWZL-tErbB1- $\Delta\alpha$ IRES-Neomycin
pWZL-tErbB2- $\Delta\omega$ IRES-Hygromycin
pWZL-tErbB2- $\Delta\alpha$ IRES- Neomycin
pWZL-tErbB3- $\Delta\omega$ IRES-Hygromycin
pWZL-tErbB3- $\Delta\alpha$ IRES- Neomycin

tEGFR-Frap- $\Delta\alpha$
tEGFR-FKBP12- $\Delta\omega$
FKBP12- $\Delta\omega$
 $\Delta\omega$ -FKBP12
Frap- $\Delta\alpha$
 $\Delta\alpha$ -Frap

Cell lines: expressing the above constructs singly and in pairwise combinations

C2C12-tErbB1- $\Delta\omega$
C2C12-tErbB1- $\Delta\alpha$
C2C12-tErbB2- $\Delta\omega$
C2C12-tErbB2- $\Delta\alpha$
C2C12-tErbB3- $\Delta\omega$
C2C12-tErbB3- $\Delta\alpha$
C2C12-tErbB1- $\Delta\omega$ /-tErbB1- $\Delta\alpha$
C2C12-tErbB2- $\Delta\omega$ /-tErbB2- $\Delta\alpha$
C2C12-tErbB3- $\Delta\omega$ /-tErbB3- $\Delta\alpha$
C2C12-tErbB1- $\Delta\omega$ /-tErbB2- $\Delta\alpha$
C2C12-tErbB3- $\Delta\omega$ /-tErbB1- $\Delta\alpha$

tEGFR-Frap- $\Delta\alpha$ + tEGFR-FKBP12- $\Delta\omega$
tEGFR-Frap- $\Delta\alpha$ + FKBP12- $\Delta\omega$
tEGFR-Frap- $\Delta\alpha$ + $\Delta\omega$ -FKBP12
tEGFR-FKBP12- $\Delta\omega$ + Frap- $\Delta\alpha$
tEGFR-FKBP12- $\Delta\omega$ + $\Delta\alpha$ -Frap

Frap- $\Delta\alpha$ + FKBP12- $\Delta\omega$
Frap- $\Delta\alpha$ + $\Delta\omega$ -FKBP12
 $\Delta\alpha$ -Frap + FKBP12- $\Delta\omega$
 $\Delta\alpha$ -Frap + $\Delta\omega$ -FKBP12

Conclusions

The initial attempts to design an assay amenable to high-throughput screening for antagonists of ErbB2 activity failed due to fundamental problems associated with the interaction system employed. In order to circumvent these limitations we rationally redesigned the system to be more stable and tolerate wider ranges of expression levels. The new system based on a 46aa mutated α -peptide was able to successfully discern inducible interactions for transmembrane receptors, cytosolic, and nuclear proteins. Further the magnitude of induction is sufficient for high throughput screening for drugs that inhibit this heterodimerization event.

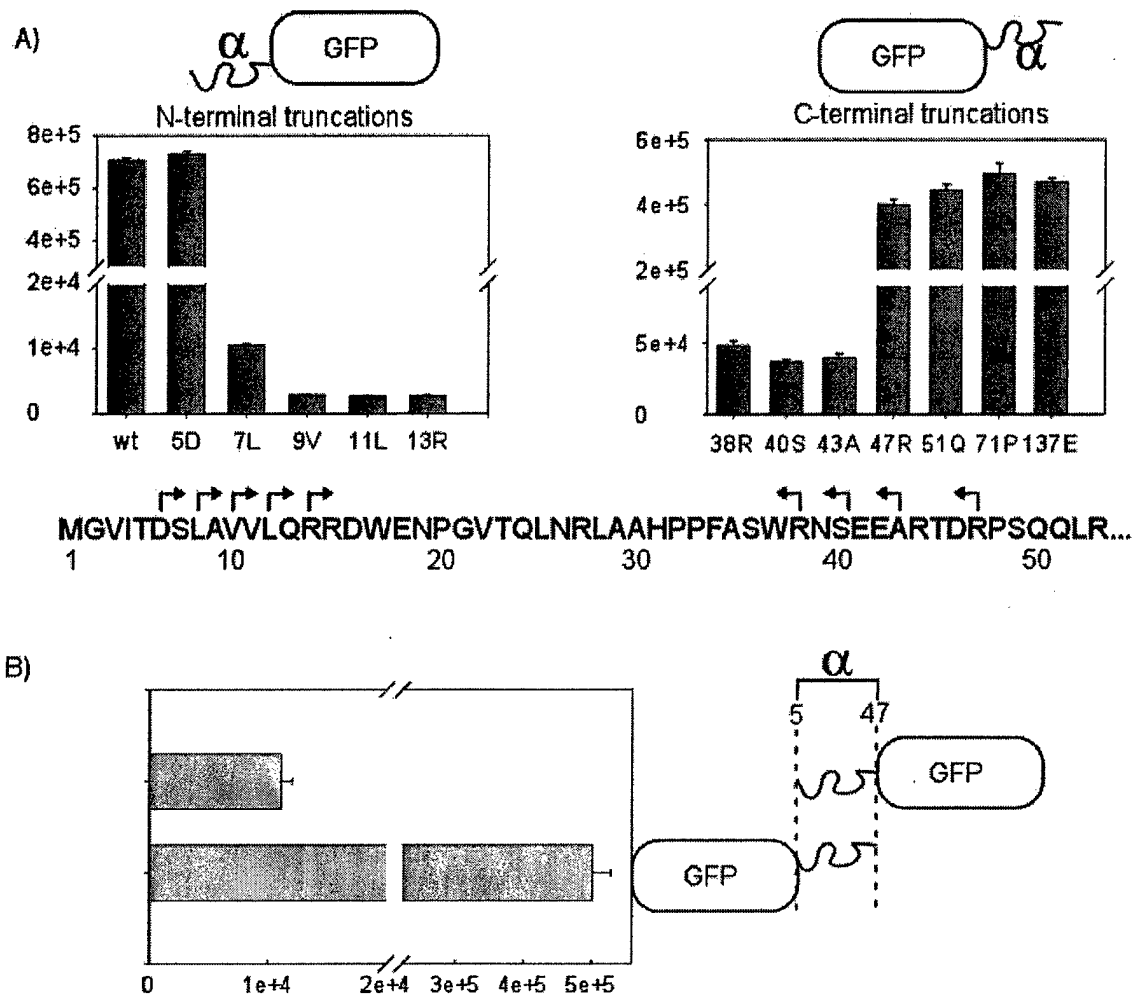


Figure 1. Design of the minimal fragment

A) (Right panel) The first 137aa of β -galactosidase were fused to the C-terminus of GFP and serially truncated. These constructs were coexpressed with the ω in C2C12 cells and assayed for β -galactosidase activity using the luminescent assay. (Left panel) aa 1-51 were fused to the N-terminus of GFP, serially truncated, then coexpressed with the ω . B) The minimal fragment was defined by combining the last C (47R) and N (5D) terminal truncations then fused to the N and C terminus of GFP. These fusions were coexpressed with the ω and assayed for enzyme activity.

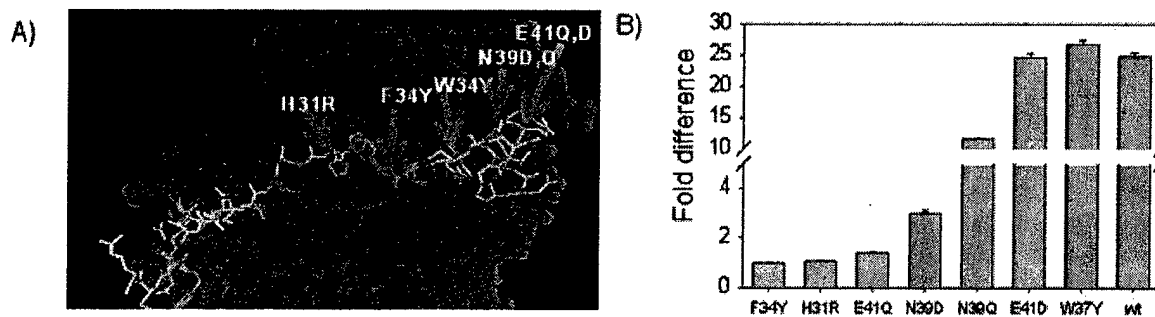


Figure 2. Mutation of the α -peptide to generate weakly complementing mutants

A) Crystal structure of wt β -galactosidase. The α is pictured in yellow and the mutations are indicated. B) Mutations were made in the α -peptide fused to the C-terminus of GFP. These constructs were transduced into cells expressing the tEGFR- ω and assayed for β -galactosidase activity (B).

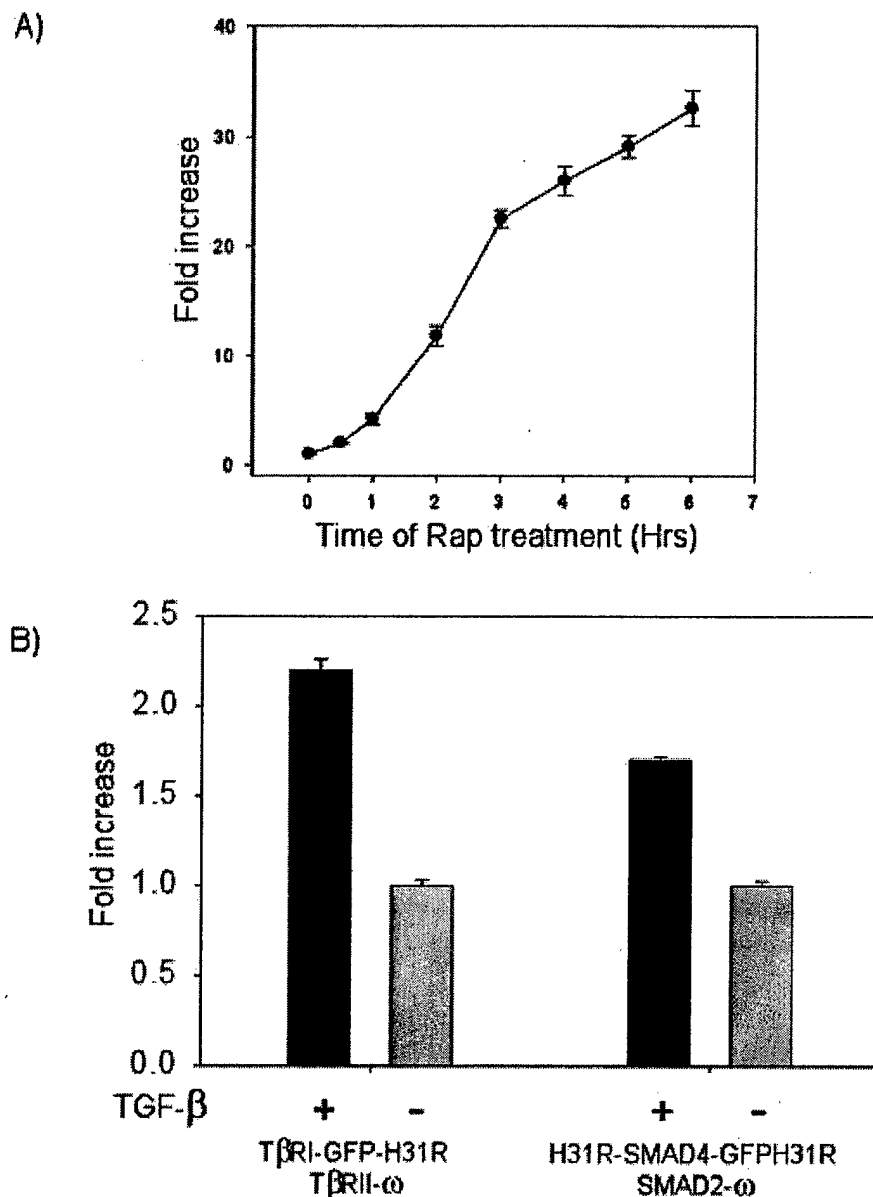


Figure 3. Protein-protein interactions monitored by β -galactosidase complementation

A) FKBP12 was fused to the N-terminus of the GFP-H31R mutant and coexpressed with the FRB- ω fusion (as previously described). Cells were assayed for β -galactosidase in the presence of 100 ng/ml rapamycin over time. B) Two protein pairs were tested for their ability to generate inducible enzyme activity, tbRI-tbRII, SMAD2-SMAD4 in the presence of TGF- β . TbRI-GFP-H31R and tbRII- ω (plasma membrane), and SMAD2- ω and SMAD4-GFP-H31R (nucleus) were tested for increases in enzyme activity in the presence of 100 ng/ml TGF- β 1 for 15 min.

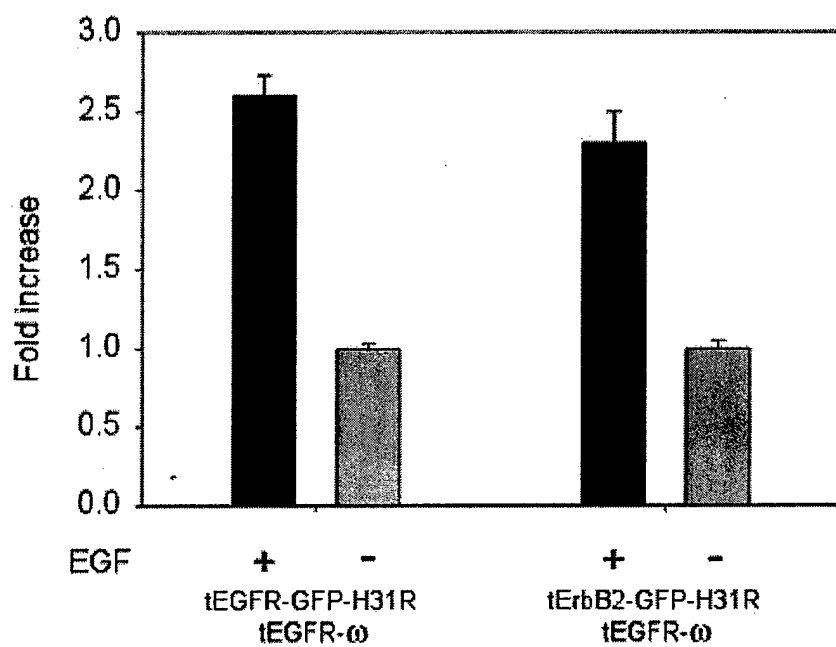


Figure 4. Homo and Heterodimerization of the EGFR and ErbB2

The homodimerization of the extracellular domains of the EGFR as well the heterodimerization of the extracellular and transmembrane domains of the EGFR and ErbB2 in the presence of 100ng/ml of EGF for 30 min. was efficiently monitored by β -galactosidase complementation.

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Appendix I – Preprint of manuscript in preparation

Protein localization and interaction monitored by modified α - complementation of β -galactosidase

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Abstract

Enzyme complementation first described in *E. coli* using β -galactosidase has been utilized in mammalian cells to monitor cell fusion, and more recently protein-protein interactions. In this work we generated a high and low affinity α -complementation strategy of β -galactosidase to monitor protein intra and intercompartmental protein translocation. Enzymatic amplification of the signal generated provides high signal to noise ratios that are unparalleled in translocation assays to date and is the first method that allows live cell-sorting based on the intracellular location of specific proteins. The low affinity α -complementation system also proved superior to previous complementation systems in monitoring protein interactions, successfully monitoring the inducible interaction of four different protein pairs including the EGFR and ErbB2.

Introduction

Directed protein movement in response to external stimuli is an essential mechanism employed by eukaryotic signal transduction pathways. Current technologies to track these events are limited to biochemical fractionation or fusion to fluorescent proteins (Lever 1980; Tsien 1998). Biochemical methods are often the most sensitive and quantitative, however they are limited by their ability to discern subcellular structures without contamination from other organelles, and the number of manipulations involved in preparing the samples makes these methods cumbersome and prone to high variability. The use of fluorescent proteins to track protein movement has positively impacted the scope and detail with which translocation events can be monitored. However the large amounts of protein necessary for efficient imaging make these experiments difficult to perform with toxic proteins and the supraphysiological levels of target protein can affect the quality of the data obtained (Balla 2002). Further, the cell-to-cell variation is high, coupled to moderately low signal to noise ratios, making the assays more qualitative than quantitative. Thus an assay combining both the localization aspects of GFP and the sensitivity and quantitative nature of biochemical assays would be ideal.

Enzyme complementation first described in *E. coli* using β -galactosidase (Ullmann 1967) has been utilized in mammalian cells to monitor cell fusion, (Mohler and Blau 1996) and more recently protein-protein interactions (Blakely, Rossi et al. 2000; Rossi, Blakely et al. 2000). Enzymatic assays have several advantages including signal amplification and a wide variety of substrates for *in vivo* and *in vitro* detection (Lis, Simon et al. 1983; Tung, Zeng et al. 2004). In this work we describe a novel assay system to monitor protein translocation based on β -galactosidase complementation. The

translocation system is comprised of two inactive fragments of β -galactosidase, the previously characterized M15 deletion mutant (ω) that is missing aa 11-41, and a short α peptide. The inert ω is localized to a specific subcellular region, and the α peptide is fused to the protein of interest. When the fusion protein moves into proximity of the ω , the ensuing complementation results in enzyme activity that can be monitored in live cells by flow cytometry, (Nolan, Fiering et al. 1988) or *in vitro* using a highly sensitive luminescent assay (Bronstein, Martin et al. 1996). Because the signal is amplified enzymatically, the translocation events can be detected at physiologically relevant expression levels of target protein. Further, the large signal generated using this technique combined with the low sample variability synergize to create a highly sensitive and quantitative measure of protein localization.

We further reasoned these mutants may be effective in monitoring protein interactions. To test this hypothesis four inducible protein-protein interactions were tested for their ability to generate enzyme activity after inducing dimerization. In all pairs tested significant changes in enzyme activity were observed. The protein pairs tested were a mixture of cell surface receptors, nuclear, and cytoplasmic proteins showing this system can detect interactions in a wide variety of cellular compartments. Markedly, the interaction system tolerated a wide range of expression levels which has traditionally hampered the utility of previous methods to monitor protein interactions (Blakely, Rossi et al. 2000).

Results

Design of the minimal fragment

α complementation of β -galactosidase involves a deletion mutant, the ω fragment (ω), that is missing aa 11-41 (Langley KE 1975). In *E. coli* and *in vitro* it is possible to complement this mutant in trans by providing the α peptide that encodes aa 3-92 (Langley KE 1975). However in mammalian cells the smallest complementing fragment was reported to contain aa 1-71 (Moosmann and Rusconi 1996). We sought to determine the smallest α peptide that would complement robustly in mammalian cells when fused to an exogenous protein at the N or C terminus. The myoblast cell line C2C12 was engineered to stably express the ω fragment by retroviral transduction. The α portion of β -galactosidase was fused to the C-terminus of GFP and deletions were made starting at aa 137 and continuing through aa 38. The products were transduced into the parental cell line expressing the ω -fragment and the cells were sorted by FACS for similar GFP expression levels. Cells were plated in 96-well dishes and assayed for β -galactosidase activity using a 1,2 dioxetane luminescent substrate (Fig. 1, right panel). From this experiment it is clear that truncations past aa 49 result in a 25-fold decrease in β -galactosidase activity. To determine which residues could be deleted at the N-terminus, aa 1-51 were fused to the N-terminus of GFP and deletions were made starting at aa 5. These constructs were similarly transduced into the parental C2C12 cell line expressing the ω -fragment. Deletions past aa 5 resulted in a 100-fold decrease in β -galactosidase activity (Fig. 1A, left panel), combining these deletions the minimal complementing fragment in mammalian cells should encompass aa 5-49. To ensure this peptide would

complement at the same level when fused to either the amino or carboxyl terminus of a protein, the peptide was fused separately to either end of GFP (Fig. 1C). When the peptide is expressed as a C-terminal fusion robust complementation is achieved, however fusion of the same peptide to the N-terminus resulted in 100-fold less activity. Thus to maintain consistency for the ensuing studies aa 5-51 (designated α) is the minimal fragment that complements to high activity in mammalian cells when expressed as a fusion protein in any orientation.

Nuclear translocation assay

In order to utilize α complementation as a method of quantitatively assessing nuclear translocation events, we designed two systems that are distinct in the localization of the ω -fragment. The gain of signal assay (Fig. 2A, left panel) localizes the ω to the nucleus through fusion to a triplet SV40 nuclear localization signal (NLS). Thus an increase in β -galactosidase activity should be observed when the target protein- α fusion moves from the cytosol to the nucleus. The loss of signal assay (Fig. 2A, right panel) localizes the ω -fragment to the plasma membrane through fusion to the extracellular and transmembrane regions of the EGF receptor (tEGFR) (Blakely, Rossi et al. 2000). This configuration results in a decrease in β -galactosidase activity when the target protein- α translocates to the nucleus (Fig. 2A).

As a test system we created a GFP- α fusion that contains the nuclear export signal from MEK (Fukuda, Gotoh et al. 1996) and a triplet SV40 NLS (Kalderon, Roberts et al. 1984). At steady-state the protein is almost exclusively located in the cytosol, but in the presence of leptomycin B (Wolff, Sanglier et al. 1997), which blocks CrmI mediated

nuclear export, the protein is retained in the nucleus (Fig. 2B, left panel). The GFP- α fusion was transduced separately into C2C12 myoblasts expressing either the ω -NLS or the tEGFR- ω construct. In the gain of signal assay, addition of leptomycin B for 2 hours resulted in a 3.5-fold increase in β -galactosidase activity assayed using the luminescent substrate. Similarly, addition of leptomycin B to the loss of signal assay resulted in almost a 5-fold decrease in β -galactosidase activity (Fig. 2B, right panel). These results demonstrate that complementation systems can be used to monitor protein translocation events in mammalian cells.

To test the applicability of the system to a physiological translocation event we fused the glucocorticoid receptor to the amino-terminus of the GFP- α fusion. The glucocorticoid receptor is a nuclear steroid hormone receptor that undergoes a robust nuclear translocation in response to treatment with various hormones including dexamethasone (Ogawa, Inouye et al. 1995). This construct was stably introduced into both the ω -NLS and tEGFR- ω cell lines then assayed for changes in β -galactosidase activity upon treatment of the cells with dexamethasone using the luminescent β -galactosidase substrate. Addition of 1 μ M dexamethasone for 3 hours to the gain of signal assay resulted in a 3.5-fold induction of β -galactosidase activity, while the loss of signal assay showed a slightly better signal to noise ratio of approximately 5-fold (Fig. 3A). The ability to maintain complementation efficiency when fused to the large fusion protein (GR-GFP) suggests that complementation can take place with proteins at least 120 KD in size.

Although differences in β -galactosidase activity are discernible as early as 30 minutes of treatment (100% for the gain of signal assay, and 25% for the loss of signal

assay) visualization of the translocation event by GFP fluorescence shows that most of the protein has translocated by this time (ref) (data not shown), however the differences in β -galactosidase activity continue to accumulate over the next three hours. α complementation in vitro using purified proteins takes 30-60 minutes to reach equilibrium (Ullmann 1967) thus we believe the lag in β -galactosidase activity to be due at least in part to the time of competent enzyme formation (gain) and breakdown (loss). Importantly, the dose response assayed over time demonstrates the ability of the system to discern different levels of stimulus (Fig. 3B). Consistently over a period of four hours, the gain of signal assay is able to significantly detect 10-fold differences in dexamethasone concentration over a 10^3 -fold range. The loss of signal assay shows a higher sensitivity to low concentrations of dexamethasone, yet maintains the ability to differentiate between concentrations ranging from 0.1 to 10 nM.

Assaying protein movement through enzyme complementation permits the use of a wide variety of substrates and detection methods, including several fluorescent β -galactosidase substrates. The GR-GFP- α cell lines were assayed in the presence and absence of dexamethasone for 3 hours using the fluorescent β -galactosidase substrate DDAO (Fig. 3C). Both systems showed a 5-10 fold change in β -galactosidase activity in the presence of dexamethasone when assayed in live cells by flow cytometry. Significantly, this is the first report of flow cytometry being used to distinguish cells based solely on the location of an intracellular protein.

α -mutants with diminished complementation capacity

The α peptide used in the nuclear translocation assay has the capability of spontaneously restoring enzyme activity when placed in the same cellular compartment as the ω . Although this method is ideal for proteins that can be separated from the ω by a physical barrier, a system to identify translocation events within the same cellular compartment would benefit from α peptides that bind the ω with lower affinity. β -galactosidase is active only as a tetramer. The crystal structure of β -galactosidase shows that residues 5-28 of the α peptide are involved in dimerization of two monomers while residues 31-41 are buried within the ω fragment. We reasoned that mutation of the buried residues would decrease the ability of the α peptide to dock with the ω while maintaining its ability to mediate tetramer formation.

We engineered several point mutations spanning residues 31-41 into the α fused to the carboxyl terminus of GFP (Fig. 4A). These constructs were transduced into the tEGFR- ω cell line, sorted for similar amounts of GFP expression, and then assayed for β -galactosidase activity using the luminescent assay. Of the seven mutations, five were found to decrease complemented enzyme activity from 2-30-fold (Fig. 4B).

Plasma membrane translocation system

Creation of a plasma membrane translocation assay required expression of the ω at the membrane which was achieved using the tEGFR- ω construct. When the target protein- α fusion moves from the cytosol to the membrane, the local increased concentration of α peptide should drive complementation as illustrated in Figure 4B. To

determine which of the mutants would work best in this type of assay we fused the C1A domain from PKC γ to four of the GFP- α point mutants that complemented to varying degrees, H31R, E41Q, N39D, and N39Q. The C1A domain has been shown to efficiently translocate to the plasma membrane when exposed to phorbol esters such as PMA (Snoek, Rosenberg et al. 1986) (Fig. 4C). The fusion proteins were expressed in NIH 3T3 cells expressing the tEGFR- ω , and assayed for increased complementation in the presence of PMA for 30 minutes. The fold induction of the various mutants was inversely proportional to the background activity for all of the mutants tested as well as the wild type peptide which showed less than a 10% increase in β -galactosidase activity (Fig. 4A). The H31R mutant, which had the lowest background activity of the mutants tested, showed a remarkable 7-fold induction of β -galactosidase activity in the presence of PMA.

The translocation of the C1A domain in response to PMA stimulation has been extensively characterized by fusion to GFP (Oancea, Teruel et al. 1998; Wang, Bhattacharyya et al. 1999). The largest increase in fluorescence at the plasma membrane in response to PMA was reported to be 70% and the lowest dose of PMA that was detectable using these methods was between 10 and 100 nM. Using β -galactosidase complementation we are able to achieve 700-1000% increases in enzyme activity in the presence of PMA, and detect as little as 1 nM PMA in the medium (Fig. 4D). Like the nuclear translocation assay, single cell analysis by flow cytometry in live cells using the DDAOG fluorescent substrate shows a large increase in fluorescence upon induction of translocation (Fig. 4E). Importantly, the requirement of the ω fragment for complementation ensures that enzyme activity is only generated where the ω -fragment is

localized. Thus the assay is measuring increases in protein concentration at a specific location in the cell.

To test whether the system was robust enough to monitor translocation events in response to physiologically relevant stimuli, we applied this system to the translocation of the PH domain of AKT. The AKT PH domain binds PI 3,4,5 trisphosphate as well as PI 3,4 bisphosphate (Franke, Yang et al. 1995; Marte and Downward 1997; Gray, Van Der Kaay et al. 1999) which are generated at the plasma membrane in response to various stimuli including peptide growth factors (Kontos, Stauffer et al. 1998; Haugh, Codazzi et al. 2000) and cellular stresses (Fujii, Ohtsubo et al. 1999; Van der Kaay, Beck et al. 1999). The PH domain of AKT was fused to the GFP- α chimera and expressed in 3T3 fibroblasts harboring the tEGFR- ω protein. The cells were stimulated under various conditions and the translocation of the PH domain was assayed in 96-well dishes by a luminescent measure of β -galactosidase activity. Osmotic shock showed the slowest, but largest increase in β -galactosidase activity (8-fold), followed by PDGF (5-fold), and insulin (2.5-fold), which agrees with biochemical quantifications using radioactive labeling and quantification of phospholipids (Gray, Van Der Kaay et al. 1999; Van der Kaay, Beck et al. 1999). However, an advantage of using the PH domain as a sensor in the β -galactosidase system is that only increases at the plasma membrane generate increases in enzyme activity due to the localization of the ω . Further, the ability to perform these assays in high-throughput format makes it possible to also perform detailed time course and dose response experiments.

The high signal to noise ratio and low standard deviations of the enzyme complementation system combine to generate a highly sensitive and quantitative assay

for physiological events. This is most evident when the stimuli are applied sequentially. Figure 5B shows a time course of stimulation with each of the four stimuli. Insulin is applied first, then PDGF, followed by sorbitol. After each stimulation the β -galactosidase complementation system registers sequential increases in activity. The shapes of each curve are dependent on the stimulation used, indicating that the rates of phospholipid generation are reflected in the magnitude and rate of change in β -galactosidase activity.

Originally we planned on imaging the plasma membrane translocation events using the fluorescence of the GFP molecule fused to the α fragment. However the toxicity associated with stable expression of these domains (Balla 2002) prevented us from expressing enough protein to image by conventional confocal microscopy. We quantified the amount of fusion protein expressed by immunoblotting the cell lines used in the β -galactosidase assays with a GFP antibody. The signal was compared to known amounts of purified GFP (Fig. 6). The amount of fusion protein expressed per μg was calculated to be approximately 50 $\text{pg}/\mu\text{g}$ of protein for GrpI and 80 $\text{pg}/\mu\text{g}$ for the AKT PH domain or 1 in 12-20,000th of total protein. The C1A domain was undetectable by Western blot. Assuming a 4 pL volume for 3t3 cells, the expression level of these domains that were readily assayed by β -galactosidase activity is 2-16 nM.

Modified α -complementation used to monitor protein interactions

Another method of increasing the dimerization of a particular protein pair is to decrease their dissociation constant through direct interaction. Due to the low background enzyme activity when the H31R mutant is coexpressed with the ω -fragment

we reasoned that the modified α -complementation system to monitor subcompartmental translocation could be applied to protein interactions. To test this hypothesis, we fused the H31R mutant to FKBP12 and FRB to the N-terminus of the ω . FKBP12 and FRB have an undetectable affinity for one another, however when the drug rapamycin is present the two proteins heterodimerize efficiently. As shown in Figure 7 the addition of rapamycin to C2C12 cells expressing both fusion constructs results in marked increases in enzyme activity.

Although this demonstrates the utility of the system for protein interactions, the FKBP12-FRB pair is unlike a typical protein interaction due to its low background (-rapamycin) and high affinity (+rapamycin) states. To determine whether the system was robust enough to monitor endogenous protein interactions we engineered four protein pairs that inducibly dimerize in a range of cellular compartments including the cytosol, nucleus, and constrained within the plasma membrane. Figure 8 shows that all protein pairs tested achieved significant interaction-dependent activity in the presence of the appropriate stimulus. The previously described system to monitor protein interactions based on β -galactosidase was extremely sensitive to fusion protein concentration such that clones of stable populations had to be screened in order to find cells that respond. The new system tolerated a wide range of expression levels and provided measurable increases in enzyme activity in heterogeneous populations. This ability vastly decreases the time invested in developing each assays and permits the comparison of cell lines expressing different protein pairs.

Discussion

Protein translocation is essential for mammalian cells to effect cellular responses, and convey information intracellularly. The use of GFP fusion proteins to track protein movement has revolutionized the ability to gather data regarding these actions and has been particularly useful in studying real-time kinetics of protein movement. However the difficulties associated with quantification of these events, such as small increases in fluorescence, high cell-to-cell variability, and the necessity for high expression levels of the fusion protein, prohibit its use in certain applications and limit the data to mainly qualitative measurements (Balla 2002).

In this work we present a novel method of quantitatively measuring protein localization that offers several advantages over either fluorescent protein or biochemical assays. The wide variety of substrates available makes this system amenable to histological, flow cytometry, and high-throughput screening applications. The peptide that is fused to the protein of interest is only 46aa, in comparison with 250aa for GFP, reducing the chances that this fusion will affect protein function. Because both subunits are necessary for complementation, efficient localization of the ω to a specific cellular location ensures signal generation solely from this region. The assay can be performed in any cell type, including non-adherent cells that are difficult to image using fluorescent proteins.

The complemented enzyme processes many molecules of substrate thus amplifying the signal obtained from the translocation event. This permits the detection of fewer numbers of molecules than microscopy based assays allowing the studied protein to be expressed at physiologically relevant concentrations. Such an assay is ideal for

toxic proteins or those localized by a binding partner that is not exogenously expressed. A further advantage of this amplification is to provide a high signal to noise ratio. The robust signal coupled to the low sample variability, generates a highly sensitive and quantitative assay for protein translocation that is applicable to all cell types in a high-throughput screening format.

The major drawback of this system is that the signal generated by enzyme activity is secondary to the translocation event, thus it is not suitable for studying processes in real time. The nuclear translocation assay showed poor kinetics. Using high concentrations of dexamethasone, complete translocation of the glucocorticoid receptor occurs in 10-15 minutes (Htun, Barsony et al. 1996), however the signal measured from β -galactosidase activity although detectable at 15 minutes, continued to increase over the next 180 minutes. By contrast the kinetics of the plasma membrane system were more predictable. The time of signal generation for the PH domains, 80% max in 10-15 minutes, closely resembled those reported for GFP fusions (80% max in ~10 min minutes) (Haugh, Codazzi et al. 2000).

Perhaps most importantly for applications that may utilize this technique, is the ability to accurately reflect levels of input and output in different systems. We showed using the glucocorticoid receptor in the nuclear translocation assay that enzyme complementation accurately described the ligand concentration at 10-fold intervals over a 1000-fold range. Even more sensitive was the C1A domain in the membrane translocation assay, detecting as little as 0.25 nM PMA (30% increase) and saturating at 800 nM PMA (1000% increase). In a more physiologically relevant setting, the enzyme complementation system correctly ordered the stimuli according to their ability to

generate phospholipids, sorbitol>PDGF>insulin through monitoring the translocation of the AKT PH domain. Further, significant increases in phospholipid production were also apparent when the stimuli were added sequentially.

This is the first report of enzyme complementation being used to study protein localization. We designed these assays using the β -galactosidase enzyme however it should be possible to adapt the other enzyme complementation systems published to study protein interactions, such as β -lactamase (Galarneau, Primeau et al. 2002; Spotts, Dolmetsch et al. 2002; Wehrman, Kleaveland et al. 2002), DHFR (Galarneau, Primeau et al. 2002), luciferase (Paulmurugan, Massoud et al. 2004), and GFP (Hu, Chinenov et al. 2002). One of the more exciting prospects for these technologies is that because signal generation is dependent on protein contact, the complementation system may be able to detect changes that would not be discernible by conventional or electron microscopy. Moreover, diffuse cellular structures or locations that are difficult to visualize using fluorescent proteins are candidates for this technology.

The same H31R α -peptide mutant was tested for its ability to monitor protein interactions *in vivo* with five separate interactions. Two of the protein interactions, SMAD2-SMAD4 and EGFR-ErbB2, failed to produce measurable increases in β -galactosidase activity using the previously developed interaction systems and the t β RI-RII interaction gave only a marginal increase ~20% (T. Wehrman unpublished results). However all performed well using the H31R modified α -complementation system. Significantly, although the EGFR is known to phosphorylate and activate ErbB2, heterodimerization of the extracellular domains as purified proteins, (Ferguson, Darling et al. 2000) or ¹²⁵I labeling and crosslinking, failed to show an inducible interaction

(Johannessen, Haugen et al. 2001). The ability to detect this heterodimerization event attests to the sensitivity and utility of this technique in monitoring protein interactions. As such this newly designed system to monitor protein interactions should have broad utility in quantitatively defining signal transduction events and in high-throughput screening applications.

Materials and Methods

Plasmids and sequences

The sequences of the tEGFR da construct and *E. coli lacZ* gene were as previously described. The GFP coding sequence used was eGFP from Clontech, and the CD8 marker was a generous gift from Garry Nolan. The triplet SV40 NLS was isolated from the YFP-NLS vector (Clontech) and the NES of MAPKK, (nt 351-403, *Gallus Gallus*) was constructed using overlapping oligonucleotides, modeled after the Caspase 3 sensor (Clontech). The full length mouse glucocorticoid receptor was isolated by RT-PCR. The PH domains of PKC γ (rat aa 26-89) and AKT ph (mus aa 2-144) were PCR amplified for fusion from plasmids gifted by Tobias Meyer.

Assays and Reagents

Stock solutions were prepared for the following stimuli, and diluted to 10X in PBS immediately before use, final concentrations are listed, PDGF (30 ng/ml), insulin (10 μ g/ml), dexamethasone, sorbitol and Leptomycin B (10 nM) (Calbiochem). Hydrogen peroxide was diluted in ddH₂O and a 5X solution of Sorbitol was made from a 2 M stock into PBS.

Chemiluminescent analysis of β -galactosidase activity.

Cells were plated at 10,000/well in 100 μ l volume in white 96-well plates (Corning Costar, Acton, MA). Cells were lysed by addition of GAL-Screen substrate (Applied Biosystems, buffer B formulation), and the plates were incubated at RT for 40 minutes. Luminescence was measured in a Tropic TR717 luminometer. Flow cytometric

detection of β -galactosidase activity was achieved using DDAOG substrate (Invitrogen). Cells were trypsinized, washed twice in PBS, then incubated with DDAOG substrate (Molecular Probes) at a concentration of 300 μ g/ml at RT for 10 minutes. Cells were pelleted, resuspended in PBS and immediately analyzed by flow cytometry on a Becton Dickinson FACS Calibur. 10,000 events were collected for each sample.

Cell culture and viral transduction

The ecotropic Φ NX packaging cell line (P.L. Achacoso and G.P. Nolan, unpublished) was transiently transfected with the proviral constructs using fugene transfection reagent (Boehringer Mannheim, Indianapolis, IN). The supernatant from the transfected cells was removed 48-72 hours later and applied to C2C12 myoblasts. Polybrene was added to a final concentration of 8 μ g/ml (Sigma, St. Louis, MO). Transduced cells were selected by FACS or incubated in the appropriate antibiotic (hygromycin or neomycin; Invitrogen, Carlsbad, CA) at a concentration of 1 mg/ml. C2C12 myoblasts were grown in DMEM (Invitrogen) 20% FBS (Hyclone). 3T3 cells were maintained in DMEM high glucose containing 10% Donor serum.

Cellular Imaging

Cells transduced with the NES-GFP-NLS- α -peptide were fixed in 2% paraformaldehyde, rinsed twice in PBS and imaged on a Zeiss Axioplan microscope using a FITC filter set.

Western Blotting

3T3 cells expressing the appropriate proteins were grown to confluence in 10 cm dishes, cells were trypsinized and cell concentrations determined (Coulter Counter). The remaining cells were lysed in M-Per and the protein concentrations were determined by Bradford analysis (Bio-Rad). Lysates were mixed with NuPAGE SDS sample buffer and run on NuPAGE 4-12% Bis-Tris gradient gels (Invitrogen) and transferred to Immobilon-P (Millipore) for immunoblotting. GFP antigen was probed using a rabbit anti-GFP (Molecular Probes) (1:1000) and detected using a goat anti-rabbit secondary conjugated to HRP (1:2000) (Zymed).

Acknowledgements:

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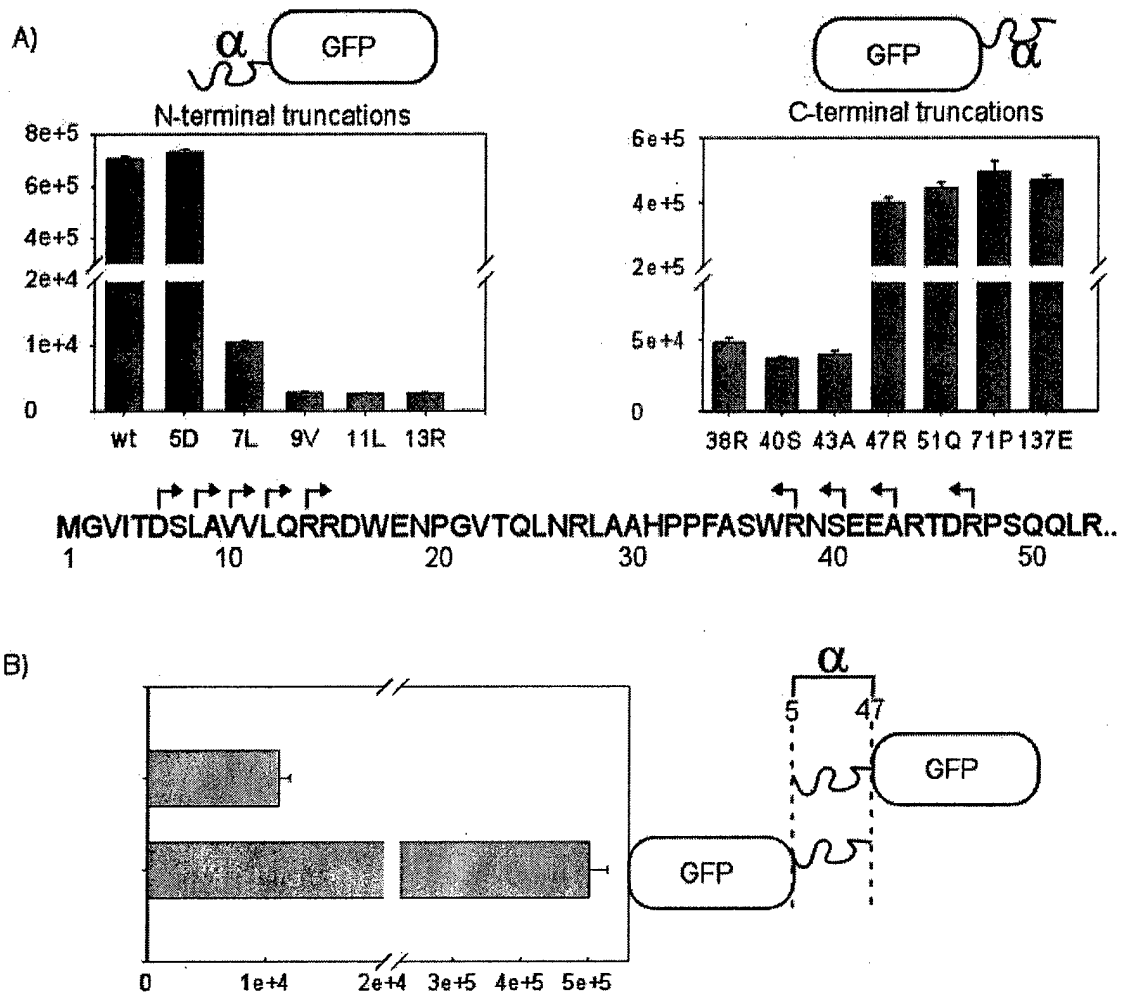


Figure 1 Design of the minimal fragment

A) (Right panel) The first 137aa of β -galactosidase were fused to the C-terminus of GFP and serially truncated. These constructs were coexpressed with the ω in C2C12 cells and assayed for β -galactosidase activity using the luminescent assay. (Left panel) aa 1-51 were fused to the N-terminus of GFP, serially truncated, then coexpressed with the ω . B) The minimal fragment was defined by combining the last C (47R) and N (5D) terminal truncations then fused to the N and C terminus of GFP. These fusions were coexpressed with the ω and assayed for enzyme activity.

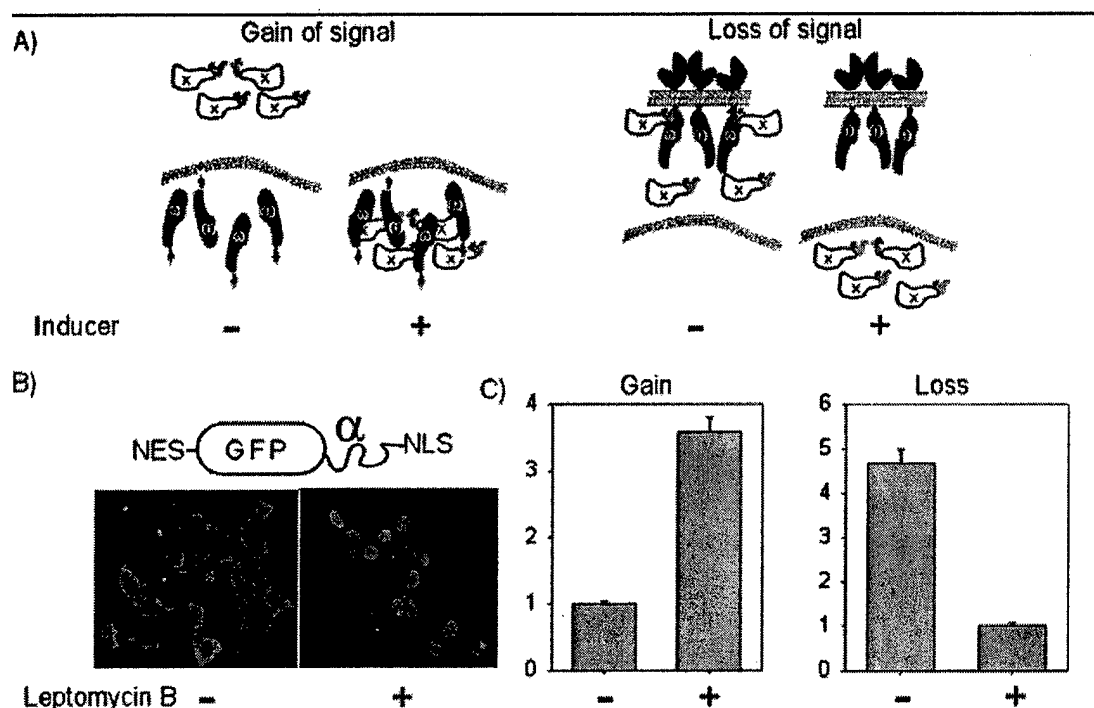


Figure 2 Enzyme complementation used to assay nuclear translocation

A) Schematic showing the design of the nuclear translocation assay. (Left panel) The ω fragment is localized to the nucleus with an NLS and the cytosolic protein of interest is fused to the minimal α peptide. Upon stimulation the α fusion moves to the nucleus and complements the ω , increasing β -galactosidase activity. (Right panel) The ω fragment is tethered to the plasma membrane using the extracellular and transmembrane regions of the EGFR. The cytosolic α -fusion complements spontaneously until stimulation to translocate to the nucleus, which results in a loss of enzyme activity. B) Nuclear translocation test system. A fusion protein consisting of the MEK NES fused to GFP, the α peptide and a triplet SV40 NLS. At steady state the protein is cytosolic (left panel). In the presence of leptomycin B the protein becomes nuclear (right panel). C) (Left panel) Gain of signal assay. The NES-GFP- α -NLS protein was coexpressed with the ω -NLS. Addition of leptomycin B for 2.5 hours results in a gain of β -galactosidase activity. (Right panel) Loss of signal assay. Transduction of cells expressing the tEGFR- ω fusion with the NES-GFP- α -NLS construct results in a loss of β -galactosidase activity in the presence of leptomycin B.

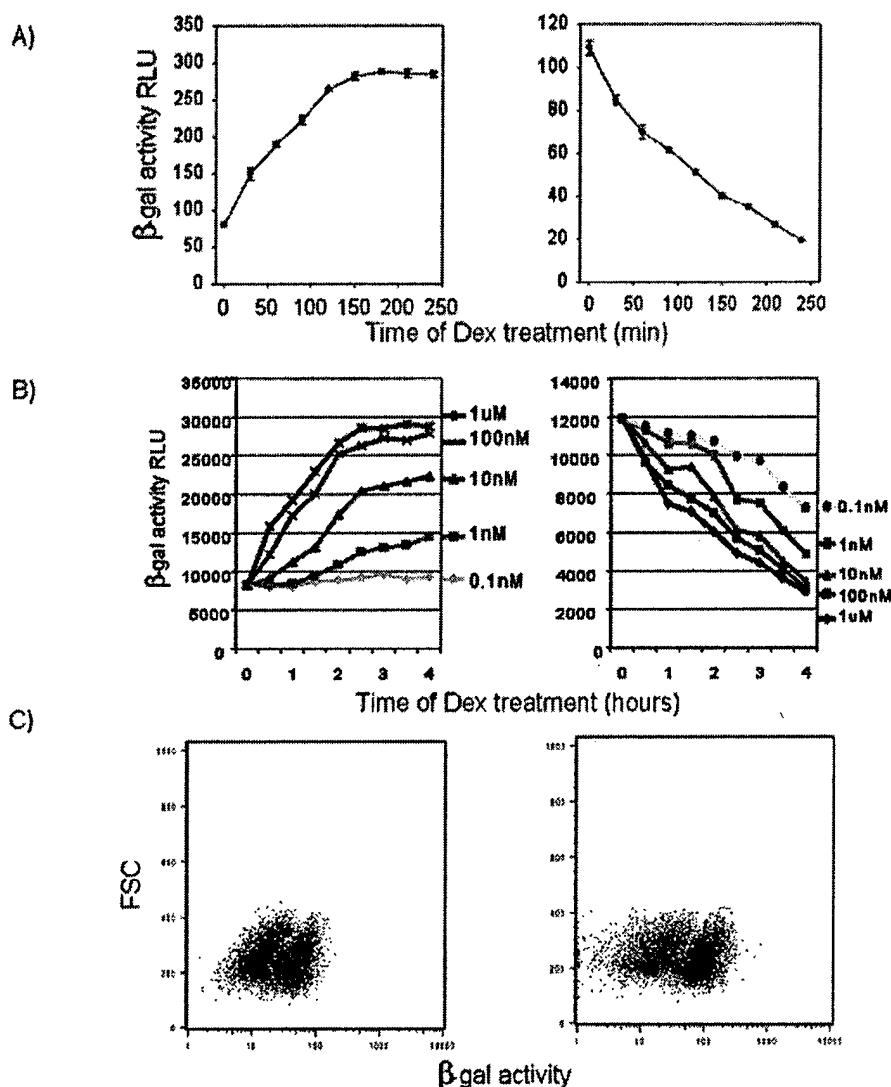


Figure 3 Nuclear translocation of the glucocorticoid receptor monitored by enzyme complementation

A) Time course. Cells expressing the GR- α fusion and either the ω -NLS (left) or tEGFR- ω (right) were assayed for β -galactosidase activity after treatment with 1 μ M dexamethasone for the indicated times. B) Dose versus time. Cell lines from A were treated with varying concentrations of dexamethasone and assayed over time. C) Protein localization monitored by flow cytometry. The GR- α cell lines were treated with 1 μ M dexamethasone (red) for 3 hours then stained with the fluorescent β -galactosidase DDAO substrate and analyzed by flow cytometry.

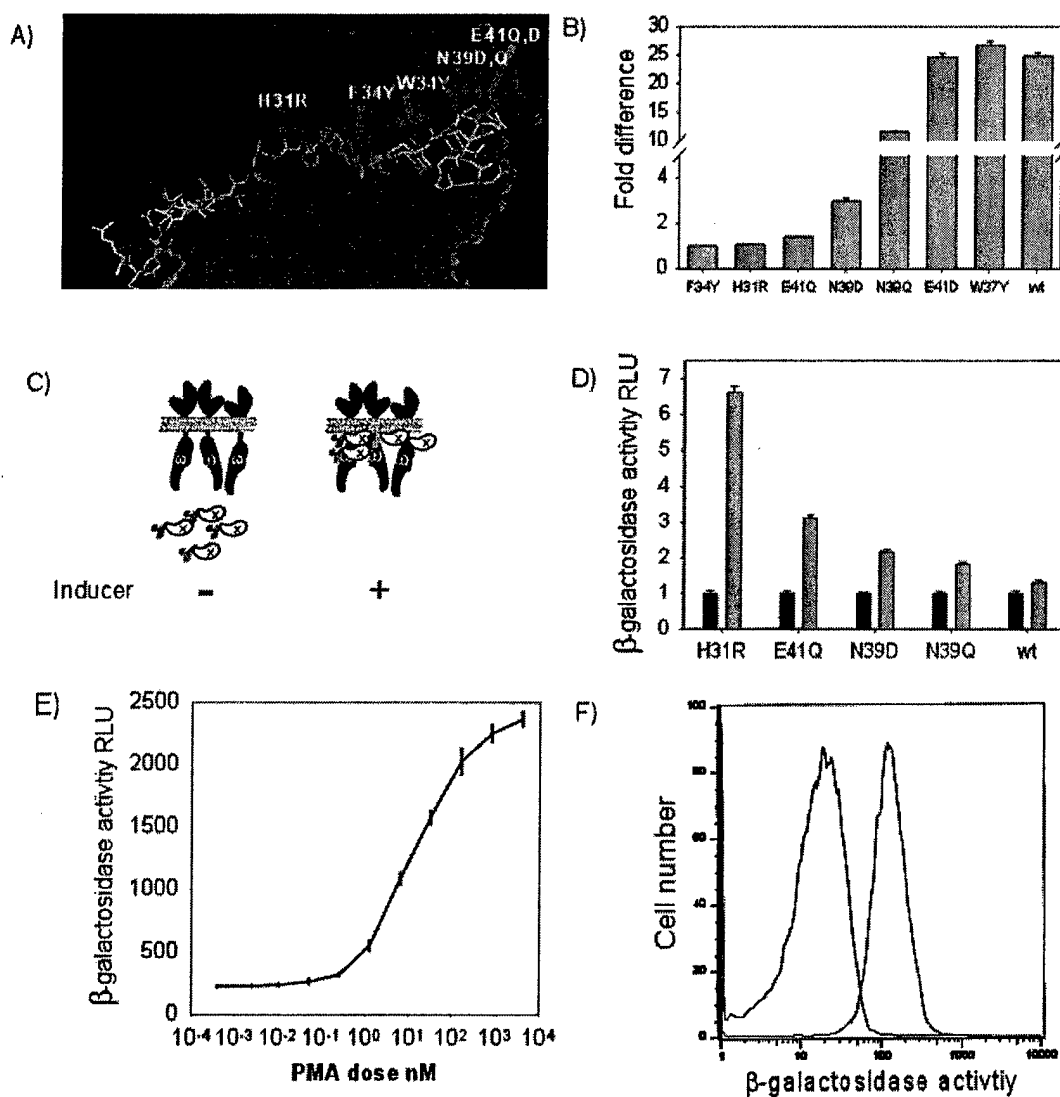


Figure 4. Mutation of the α -peptide to generate weakly complementing mutants

A) Crystal structure of wt β -galactosidase. The α is pictured in yellow and the mutations are indicated. B) Mutations were made in the α -peptide fused to the C-terminus of GFP. These constructs were transduced into cells expressing the tEGFR- ω and assayed for β -galactosidase activity.

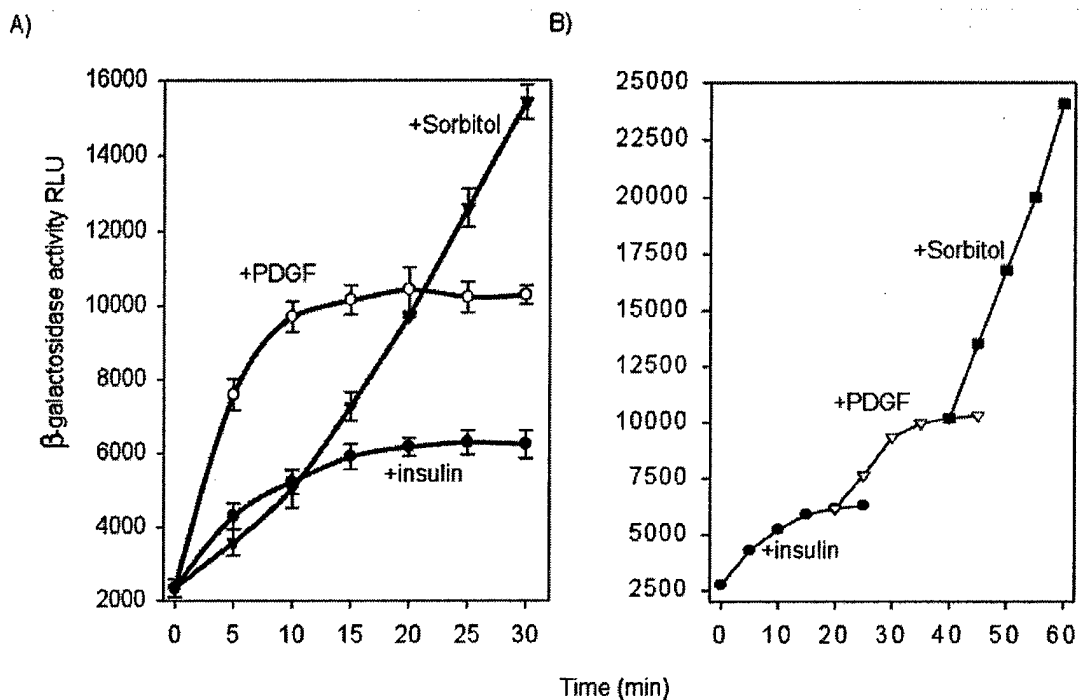


Figure 5 Translocation of the AKT PH domain monitored by β -galactosidase complementation

A) The AKT-GFP- α fusion protein was transduced into 3T3 cells expressing the tEGFR- ω fusion. The cells were treated with 50 ng/ml PDGF, insulin, or sorbitol for the indicated times and assayed for β -galactosidase activity using the luminescent substrate.

B) Sequential stimulation. The cells used in A were sequentially stimulated with insulin, PDGF and sorbitol and assayed for β -galactosidase activity.

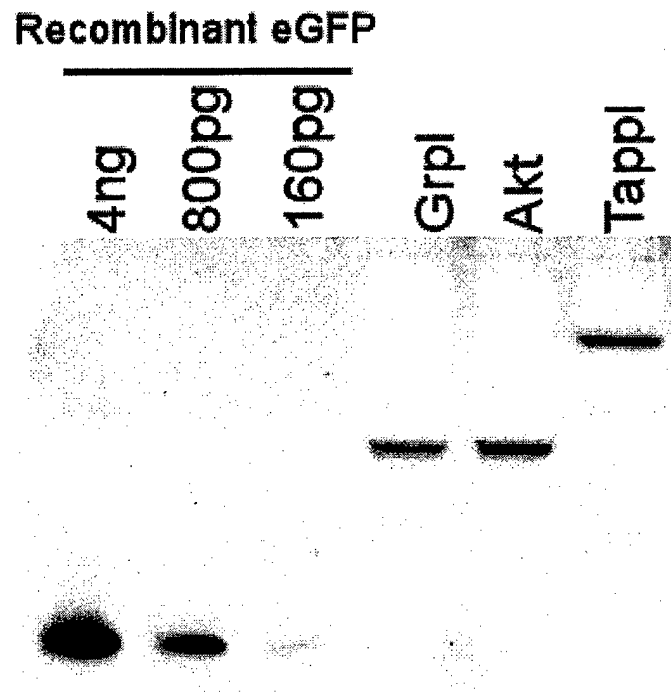


Figure 6 Low levels of expressed proteins can still be efficiently detected using enzyme complementation

Total cell lysates (6.5 μ g) from the lines expressing the indicated PH domains fused to the GFP- α chimera that were used in Figure 5 were immunoblotted for expression of the fusion protein. A titration of recombinant GFP was included to quantitate the levels of protein.

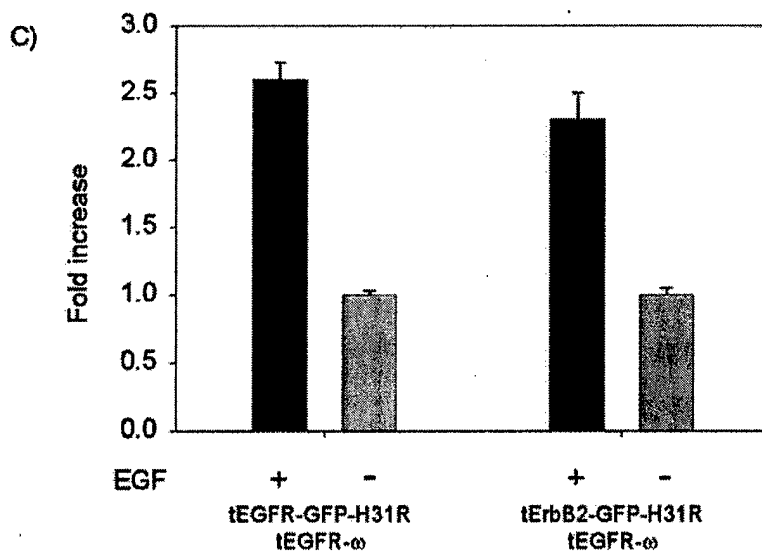
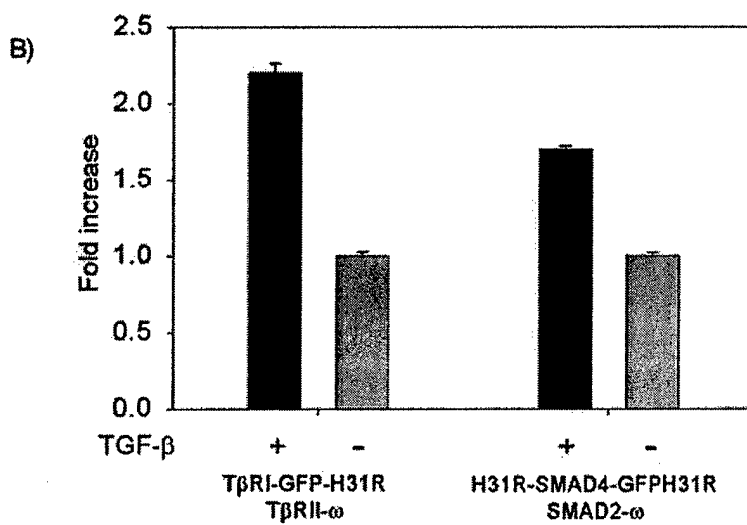
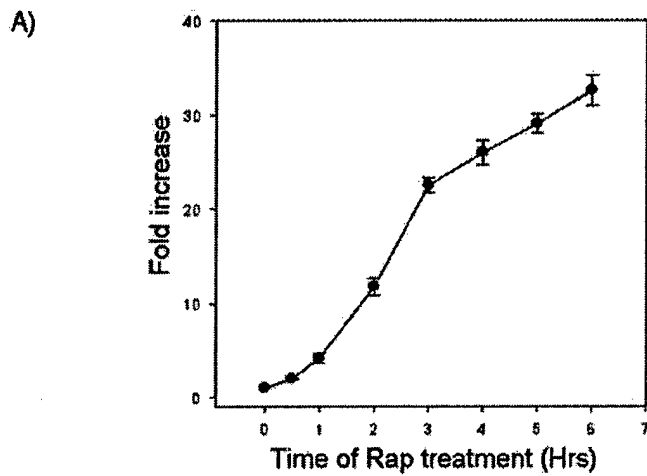


Figure 7 Protein-protein interactions monitored by β -galactosidase complementation

A) FKBP12 was fused to the N-terminus of the GFP-H31R mutant and coexpressed with the FRB-w fusion (as previously described). Cells were assayed for β -galactosidase in the presence of 100 ng/ml rapamycin over time. B) Four protein pairs were tested for their ability to generate inducible enzyme activity, tbRI-tBRII, SMAD2-SMAD4, tEGFR-tEGFR, and tEGFR-tErbB2. TbRI-GFP-H31R and tbRII-w (plasma membrane), and SMAD2-w and SMAD4-GFP-H31R (nucleus) were tested for increases in enzyme activity in the presence of 100 ng/ml TGF- β 1 for 15 min. The homodimerization of the extracellular domains of the EGFR as well the heterodimerization of the extracellular and transmembrane domains of the EGFR and ErbB2 in the presence of 100ng/ml of EGF for 30 min. was efficiently monitored by β -galactosidase complementation in the presence of X-gal.

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